RESEARCH COMMUNICATION

Nitric oxide rapidly scavenges tyrosine and tryptophan radicals

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By utilizing a pulse-radiolytic technique, we demonstrate for the first time that the rate constant for the reaction of nitric oxide ($^{\bullet}NO$) with biologically relevant tyrosine and tryptophan radicals (Tyr$^{\bullet}$ and Trp$^{\bullet}$ respectively) in amino acids, peptides and proteins is of the order of $(1–2) \times 10^9$ $M^{-1} \cdot s^{-1}$. We also show that $^{\bullet}NO$ effectively interferes with electron-transfer processes between tryptophan and tyrosine residues in proteins subjected to pulse radiolysis. The near diffusion-controlled rates of these reactions, coupled with the increasingly recognized role of protein radicals in enzyme catalysis and oxidative damage, suggest that Tyr$^{\bullet}$ and Trp$^{\bullet}$ are likely and important targets for $^{\bullet}NO$ generated *in vivo*.

INTRODUCTION

Nitrogen monoxide (nitric oxide, $^{\bullet}NO$) is endogenously produced in a variety of mammalian cells by both constitutive and inducible forms of nitric oxide synthase (NOS) [1]. $^{\bullet}NO$ is an important mediator of a variety of diverse biochemical and physiological processes, including signal transduction, neurotransmission, smooth muscle relaxation, platelet inhibition, blood pressure modulation, immune system control, macrophage-mediated cytotoxicity, learning and memory [2]. The mechanisms by which many of these events proceed, however, are only partially understood.

Unlike many free radical species, $^{\bullet}NO$ displays highly selective reactivity, has a relatively long biological half-life and is capable of transmembrane diffusion due to its charge neutrality and small size. These chemical and physical properties of $^{\bullet}NO$ facilitate its activity as a cellular messenger and mediator. The major biological targets associated with endogenous and exogenous $^{\bullet}NO$ include haem-iron-, iron–sulphur- and thiol-containing proteins. $^{\bullet}NO$ can bind to iron in both haem proteins and non-haem proteins with high affinity [3], and activation of guanylate cyclase [4] and the proposed feedback inhibition mechanism of NOS [5] are mediated through this mechanism.

Although direct reaction of $^{\bullet}NO$ with most biological molecules is thought to be slow, its reaction with other free radicals is very fast. For instance, $^{\bullet}NO$ reacts extremely rapidly with superoxide radical ($O_2^{\bullet-}$) ($k = 6.7 \times 10^9$ $M^{-1} \cdot s^{-1}$) [6] to form the cytotoxic species, peroxynitrite ($ONOO^-$). In fact, at sufficiently high concentrations, $^{\bullet}NO$ can compete with superoxide dismutase for reaction with $O_2^{\bullet-}$.

Free radicals in proteins are becoming increasingly recognized as both the unwanted progression of oxidative free-radical damage [7] and as functional, catalytic components of a variety of enzyme systems [8]. In particular, tyrosine radicals (Tyr$^{\bullet}$) have been detected in a variety of enzymes [9], including ribonucleotide reductase, prostaglandin H synthase, photosystem II, galactose oxidase, and in peroxidases, haemoglobin and myoglobin after reaction with hydrogen peroxide. A tryptophan radical (Trp$^{\bullet}$) has been identified in cytochrome-*c* peroxidase [10] and in the active site of DNA photolyase [11]. In fact, charge transfer between tryptophan and tyrosine residues in proteins and peptides has been observed in pulse-radiolysis experiments [12,13] and may represent a significant process in enzyme function. In many cases, the protein radical itself may play an important role in the active site of the enzyme, as has been reported recently for prostaglandin H synthase [14]. Protein/enzyme radicals, therefore, are probable and important biological targets for $^{\bullet}NO$ that have, until now, been largely overlooked. Recently, $^{\bullet}NO$ has been shown to inactivate ribonucleotide reductase, with concomitant loss of the functional Tyr$^{\bullet}$ [15]. We report here that $^{\bullet}NO$ reacts directly with Tyr$^{\bullet}$ and Trp$^{\bullet}$ in amino acids, peptides and proteins at rates that are orders of magnitude greater than its binding to protein-bound metal ions.

MATERIALS AND METHODS

Chemicals and reagents

L-Tyrosine, L-tryptophan, *N*-acetyl-L-tyrosine, L-glycine-L-tyrosine (Gly-Tyr), L-tryptophan-L-tyrosine (Trp-Tyr), BSA (essentially fatty acid free), β-lactoglobulin (A + B), pepsin, lysozyme and ribonuclease A were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). $^{\bullet}NO$ gas (99%) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Aldrich Chemical Co. and nitrous oxide (N_2O, 99.999%) was obtained from B.O.C. (U.K.). Sodium azide (NaN_3) was from Merck (Darmstadt, Germany). All solutions were prepared with de-ionized, doubly distilled water.

Preparation of $^{\bullet}NO$ solutions

Before $^{\bullet}NO$ solutions were made, a 50 ml solution of phosphate buffer (10 mM, pH 7.4) was purged with N_2O for at least 20 min to remove O_2. $^{\bullet}NO$ gas was slowly bubbled through a scrubbing

Abbreviations used: $^{\bullet}NO$, nitric oxide; $N_3^{\bullet}$, azide radical; $ONOO^-$, peroxynitrite; $O_2^{\bullet-}$, superoxide radical; Tyr$^{\bullet}$, tyrosine radical; Trp$^{\bullet}$, tryptophan radical; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid); NOS, nitric oxide synthase; LDL, low-density lipoprotein.

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vessel which contained 5 M KOH that served to remove contaminating higher oxides of nitrogen. The N_2O-saturated buffer solution was placed in series with the purified •NO stream and continuously bubbled for 20 min to produce a saturated solution having a final •NO concentration of approximately 1.7 mM, as measured by ABTS oxidation [16]. Fresh •NO stock solutions were prepared for each series of experiments.

Pulse-radiolysis experiments

Radical reactions in amino acids, peptides and proteins were initiated by a short (20 ns), high-energy electron pulse from a linear accelerator described previously [13]. Reactions were induced in N_2O-saturated phosphate-buffered (10 mM, pH 7.4; unless otherwise noted) solutions containing the substrate (amino acid, peptide or protein) and 100 mM NaN_3. In some reactions, an aliquot (0.1–0.8 ml) of the •NO stock solution was added with a gas-tight syringe through a rubber septum into the sealed experimental apparatus containing the reaction solution. Pulse experiments were conducted within 5 min following •NO addition. No loss of •NO could be detected 20 min after addition to the solution.

Azide radicals ($N_3^{\bullet}$) are formed in aqueous solutions of sodium azide (N_3^-) within about 0.1 μs after the pulse through the following reactions:

$$H_2O \rightsquigarrow {}^{\bullet}OH, H^{\bullet}, e^-_{aq.}, H_2, H_2O_2, H_3O^+$$

$$e^-_{aq.} + N_2O + H_2O \rightarrow {}^{\bullet}OH + OH^- + N_2$$

$$^{\bullet}OH + N_3^- \rightarrow N_3^{\bullet} + OH^-$$

The generated $N_3^{\bullet}$ selectively and rapidly oxidizes tyrosine and tryptophan residues to the corresponding radicals [17]. Dosimetry was by the thiocyanate method as described previously [18]. Doses (D) were in the region of 1–5 Gy per pulse. Initial radical yields were taken to be $G(N_3^{\bullet}) = G(^{\bullet}OH) + G(e^-_{aq.}) \approx 5.6(100\ eV)^{-1}$, corresponding to a molar concentration of $G(N_3^{\bullet}) \times D \times 1.035 \times 10^{-7}$ M, and normally kept between 1 and 2 μM to ensure pseudo-first-order kinetics.

The optical path-length of the cell was 2.5 cm. Transient absorbance changes in Tyr• and Trp• were detected by kinetic absorbance spectroscopy at 405 and 510 nm respectively. The transient signals were recorded by a Tektronix digitizer and analysed by a microcomputer. The yield of radicals formed was calculated from transient absorbances using the following absorption coefficients: $A_{510}(Trp^{\bullet}) = 1.96 \times 10^3\ M^{-1} \cdot cm^{-1}$, $A_{405}(Trp^{\bullet}) = 0.4 \times 10^3\ M^{-1} \cdot cm^{-1}$, $A_{510}(Tyr^{\bullet}) = 0\ M^{-1} \cdot cm^{-1}$ and $A_{405}(Tyr^{\bullet}) = 3.2 \times 10^3\ M^{-1} \cdot cm^{-1}$ [17].

RESULTS AND DISCUSSION

The production of $N_3^{\bullet}$ in our pulse-radiolysis system allows rapid formation of Tyr• and Trp• in amino acids, peptides or proteins as follows:

$$TrpH + N_3^{\bullet} \rightarrow Trp^{\bullet} + N_3^- + H^+$$

$$TyrH + N_3^{\bullet} \rightarrow Tyr^{\bullet} + N_3^- + H^+$$

We have utilized this pulse-radiolytic technique to determine the reaction rate constant of •NO with Tyr• and Trp•. Irradiation of N_2O-saturated solutions of Gly-Tyr (10 mM) led to the rapid formation of Gly-Tyr•, as is evident from its characteristic absorbance at 405 nm, which decayed, through self-reaction presumably, to give the dimerized product [$(Gly\text{-}Tyr)_2$]. •NO increased the apparent rate of Gly-Tyr• decay, indicating a

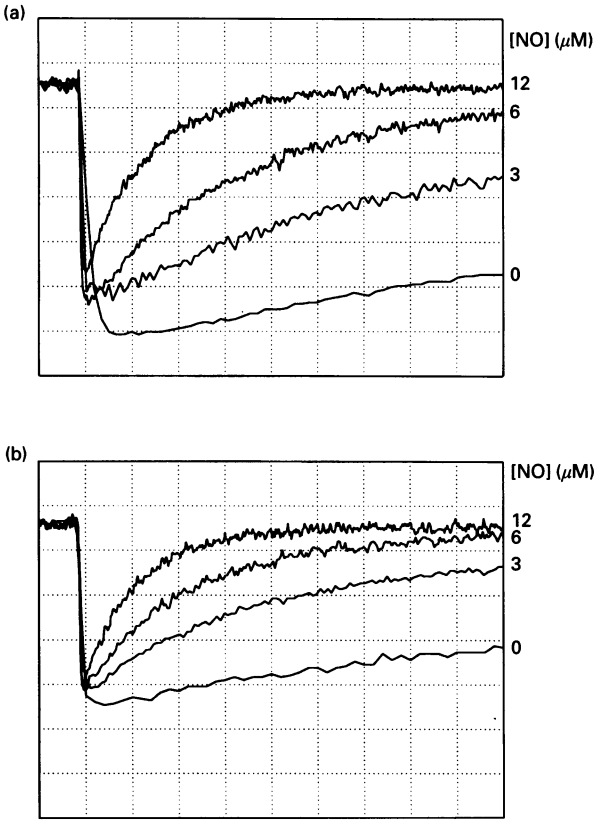

Figure 1 Effect of various •NO concentrations on the transient kinetic profiles of Gly-Tyr• and Trp•

N_2O-saturated solutions of Gly-Tyr (10 mM) and Trp (10 mM) containing N_3^- (100 mM) at pH 7.4 were subjected to a single 20 ns pulse of high-energy electrons in the presence of increasing concentrations of •NO, as described in the Materials and methods section. Representative time profiles showing the formation and decay of Gly-Tyr• (**a**) and Trp• (**b**) were measured at 405 and 510 nm respectively. The time scales are 25 μs/division at a sensitivity of 1% transmission/division.

reaction between the two radicals that was faster than the natural decay of Tyr• (Figure 1a). A linear correlation was obtained when pseudo-first-order rate constants (s^{-1}) were plotted against •NO concentration (Figure 2). Similar experiments were performed for Trp (10 mM) and the corresponding kinetic absorbance traces and first-order plot are shown in Figures 1(b) and 2 respectively. The rate constants for the reactions of •NO with Gly-Tyr• and Trp• were determined to be $1.2 \pm 0.3 \times 10^9$ and $1.4 \pm 0.2 \times 10^9\ M^{-1} \cdot s^{-1}$, respectively. Both free L-tyrosine (1 mM, pH 9.0) and *N*-acetyl-L-tyrosine (10 mM, pH 7.4) yielded similar reaction rate constants (Table 1).

We also performed the pulse experiments with a peptide containing both Tyr and Trp. At physiological pH (7.4), the reaction of $N_3^{\bullet}$ with tryptophan is about ten times faster than its reaction with tyrosine [17], which facilitates the selective initial formation of Trp•. Irradiation of Trp-Tyr (2 mM) resulted in an initial transient absorbance at 510 nm (4 μs after the pulse) which rapidly decayed to yield an absorbance at 405 nm, indicating Trp• and Tyr• formation respectively. The intramolecular electron transfer reaction in Trp-Tyr, shown below, occurs over approx. 10 μs and has a first-order rate constant of $6 \times 10^4\ s^{-1}$ [17]:

$$Trp^{\bullet}\text{-}TyrOH \rightarrow TrpH\text{-}TyrO^{\bullet}$$

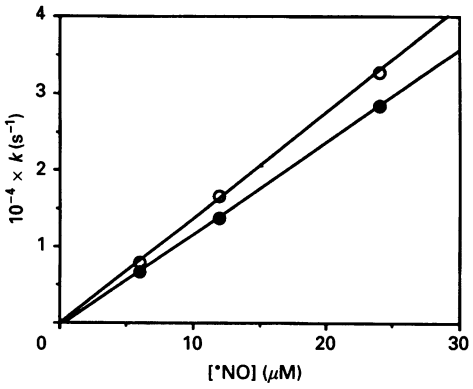

Figure 2 Plot of the pseudo-first-order rate constants for the decay of Trp• and Tyr• as a function of •NO concentration

Solutions of Trp (10 mM) and Gly-Tyr (10 mM) were subjected to pulse radiolysis in the presence of increasing concentrations of •NO, as described in the Materials and methods section and in Figure 1. Pseudo-first-order rate constants for the decay of Trp• (○) and Gly-Tyr• (●) were determined by kinetic examination of the absorption traces. Data points represent the mean of at least three different pulse experiments.

Table 1 Summary of experimentally determined reaction rate constants of •NO with Trp• and Tyr• radicals in amino acids, peptides and proteins

N_2O-saturated solutions (10 mM phosphate, pH 7.4; except where noted otherwise) of the various amino acids, peptides and proteins containing N_3^- (100 mM) and various concentrations of •NO (3–24 μM) were irradiated with a 20 ns pulse at doses of 1–5 Gy. Reaction rate constants were determined for at least four different •NO concentrations. The rate constants are expressed as the means ± S.D. of at least four different evaluations.

Reaction	Reaction rate constant, k ($M^{-1} \cdot s^{-1}$)
Tyr• + •NO (pH 9.0)	$1.0 \pm 0.3 \times 10^9$
N-Acetyltyr• + •NO	$2.0 \pm 0.4 \times 10^9$
Gly-Tyr• + •NO	$1.2 \pm 0.3 \times 10^9$
Trp• + •NO	$1.4 \pm 0.2 \times 10^9$
Trp-Tyr• + •NO	$8.7 \pm 0.4 \times 10^8$
Pepsin-Trp• + •NO	$8.3 \pm 0.6 \times 10^8$
β-Lactoglobulin-Trp• + •NO	$1.3 \pm 0.3 \times 10^9$

We have determined the rate constant for the reaction of •NO with Tyr• in Trp-Tyr to be $8.7 \pm 0.4 \times 10^8$ $M^{-1} \cdot s^{-1}$, slightly lower than that determined for Gly-Tyr or *N*-acetyl-L-tyrosine alone. Some •NO may be lost to the initially formed Trp•, and hence, may lead to a slight underestimation of the calculated rate constant.

The rate of electron transfer between Trp• and Tyr is typically much slower in proteins ($k = 10^2$–10^3 s^{-1}) and complete transfer takes between 250 and 900 μs, producing a relatively long-lived Trp• intermediate [13]. We chose to study β-lactoglobulin as a model protein because the electron-transfer processes involving Trp• and Tyr• have been extensively characterized in this protein [13]. Increasing amounts of •NO added to N_2O-saturated solutions of β-lactoglobulin (2 mg/ml) increased the apparent rate of Trp• decay accordingly, yielding transient absorbance traces similar to those shown in Figure 1 (results not shown). A rate constant of $1.3 \pm 0.3 \times 10^9$ $M^{-1} \cdot s^{-1}$ was determined for the reaction of •NO with Trp• in β-lactoglobulin. A survey of other proteins, including pepsin, lysozyme, ribonuclease A and BSA (all at concentrations of 4 mg/ml), gave slightly different, but still extremely high, reaction rate constants. For instance, the rate constant for the reaction of •NO with Trp• in pepsin was determined to be $8.3 \pm 0.6 \times 10^8$ $M^{-1} \cdot s^{-1}$. The rate constants for the reaction of •NO with Trp• in all of the other proteins studied fell into the range between that determined for pepsin and β-lactoglobulin ($k \sim 10^9$ $M^{-1} \cdot s^{-1}$).

Table 2 Effect of various concentrations of •NO on the yield of Trp• and Tyr• in β-lactoglobulin following pulse radiolysis

An N_2O-saturated solution of β-lactogobulin (2 mg/ml) containing various concentrations of •NO (0–24 μM) was irradiated as described in the Materials and methods section. The yields of Trp• and Tyr• were determined approx. 50 and 900 μs after the pulse (times at which maximal yields were reached) respectively, and were calculated using the absorption coefficients previously reported [18]. Data are expressed as the percentage of radical yield determined in the absence of •NO and are the means ± S.D. of three separate pulse experiments.

Nitric oxide (μM)	Radical yield (%)	
	Trp•	Tyr•
0	100	100
6	43 ± 6	82 ± 5
12	34 ± 5	64 ± 6
24	23 ± 3	34 ± 5

Owing to the reactions of •NO with a small fraction of the N_3• ($k \sim 10^9$ $M^{-1} \cdot s^{-1}$) and with the initially formed Trp•, it was not possible to determine accurately rate constants for reaction of •NO with the Tyr• formed by electron transfer in the proteins. However, it was clear that •NO decreased the efficiency of the electron-transfer process between Trp and Tyr. •NO decreased the yield of both Trp• and Tyr• in all of the proteins studied. Table 2 shows the decreased yields of Trp• and Tyr• in β-lactoglobulin as a function of •NO concentration. •NO had variable effects on the overall yields of these radicals in the different proteins studied. These variations in radical yield can be explained by differences in the mechanisms and rates of electron transfer, both dependent on protein structure and on the number of Trp and Tyr residues. For instance, whilst β-lactoglobulin (2 Trp and 4 Tyr residues) displays simple first-order decay of Trp•, the decay of Trp• in pepsin (5 Trp and 16 Tyr residues) is composed of at least two kinetically distinct first-order processes [18]. Similar phenomena have been observed in pulse-radiolysis experiments with purified ribonucleotide reductase [19], which shows a biphasic decay of the Trp•. Our results suggest that •NO interferes with electron-transfer processes in proteins where Trp• and Tyr• are involved as redox intermediates.

We propose that •NO reacts with these species by a radical–radical addition reaction as illustrated in Figure 3. Tyr• reacts extremely rapidly with nitrogen dioxide (•NO_2) ($k = 3 \times 10^9$ $M^{-1} \cdot s^{-1}$) to form 3-nitrotyrosine [20]. By analogy, we suggest that a probable major product of the reaction between •NO and Tyr• is 3-nitrosotyrosine, although the formation of the O-nitroso product is also possible due to the delocalization of the radical in the aromatic system. We expect •NO to react with Trp• to form the N-nitroso species. The formation of C- and O-nitroso adducts from reaction of •NO with a variety of phenols has been reported previously [21]. The reaction was found to be reversible, as the nitroso adduct slowly dissociates back to a phenoxyl radical and •NO. No observable products of these reactions could be identified by spectral scanning during our pulse experiments, most likely because of their inherent instability

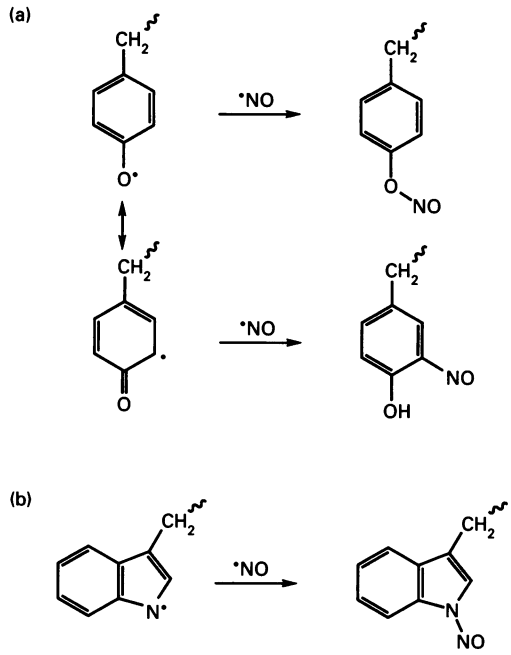

Figure 3 Proposed mechanisms for the reaction of 'NO with Tyr' (a) and Trp' (b) in proteins and free amino acids

under the experimental conditions [22]. In the presence of oxidants such as H_2O_2 or HOCl at sites of inflammation *in vivo*, the 3-nitrosotyrosine product could theoretically be oxidized to 3-nitrotyrosine, a reaction typical of aromatic nitroso compounds [22]. These reactions suggest an alternative mechanism for the formation of 3-nitrotyrosine *in vivo* in human acute lung injury, atherosclerosis and rheumatoid arthritis, which has recently been attributed to $ONOO^-$ formation [23–25].

The very rapid radical–radical reactions of 'NO that we report here are analogous to those observed for reaction of 'NO with $O_2^{\cdot-}$, organic peroxyl radicals (ROO') and other inorganic radicals, yielding rate constants in the range $1–6 \times 10^9$ $M^{-1} \cdot s^{-1}$ [6,26,27]. Such radical–radical termination reactions may sometimes allow 'NO to act as an endogenous biological antioxidant. This is exemplified by the effectiveness of excess 'NO in preventing the peroxidation of lipids by serving as a potent scavenger of lipid alkoxyl (LO') and peroxyl (LOO') radicals [28]. Recently, Tyr' generated by a myeloperoxidase system has been proposed to act as an endogenous physiological catalyst for the initiation of metal ion-independent peroxidation of low-density lipoprotein (LDL) [29]. The importance of phenoxyl radicals *in vivo* is underscored by their nearly 300-fold greater reactivity compared with organic peroxyl radicals [30]. 'NO, then, might serve as an important antioxidant in the vasculature by scavenging endogenous free Tyr'. It is likely that 'NO could also scavenge α-tocopheroxyl radicals, thereby terminating potential radical chain reactions and perhaps recycling α-tocopherol. Indeed, 'NO appears to be anti-atherogenic [31], and one of its effects may be to inhibit LDL oxidation through these reaction pathways. 'NO would also be expected to interfere with processes associated with Tyr'-mediated protein cross-linking [32]. Maximal production of 'NO in tissues has been shown to reach a steady-state concentration of approx. 1–2 μM [33], which is orders of magnitude less than the levels of endogenous antioxidants such as ascorbate and urate. However, 'NO scavenges Tyr' and Trp' at least 100 times faster than these antioxidants; the rate constants for the reaction of ascorbate and urate with Tyr' are 1.1×10^7 and 5.4×10^6 $M^{-1} \cdot s^{-1}$ respectively [34]. Moreover, because of its small size and charge neutrality, 'NO is probably capable of diffusing to cellular compartments and regions of proteins inaccessible to other endogenous antioxidants.

It is argued that 'NO binding to both haem- and non-haem-iron in proteins is central to its activation and inactivation of a variety of enzymes, including guanylate cyclase and NOS [4,5], as well as the inhibition of lipoxygenase-, cyclooxygenase- and haemoglobin-mediated lipid peroxidation [35]. However, protein radicals residing on Tyr and Trp residues are emerging as important components of a variety of enzymes and peroxide-activated haem-proteins. In many cases, these protein radicals are spatially quite near to the metal centre of the protein; 5.3 Å (1 Å = 0.1 nm) in the case of ribonucleotide reductase [36]. The rate constants for the reactions of 'NO with the iron in various haem proteins are in the range $10^4–10^5$ $M^{-1} \cdot s^{-1}$ (ferric states) and approximately 10^7 $M^{-1} \cdot s^{-1}$ (ferrous states) [37], which are orders of magnitude lower than that for the reaction of 'NO with Tyr' or Trp' (summarized in Table 1). We argue, therefore, from kinetic considerations, that in the microenvironment of a metal–protein radical couple, physiological levels of 'NO will favour reaction with the protein radical (Tyr' or Trp') over reaction with the metal centre. Most recently, 'NO has been shown to rapidly and reversibly inactivate purified ribonucleotide reductase by iron-independent mechanisms involving cysteine nitrosylation and possible Tyr' quenching [38]. Our results prove that 'NO is capable of direct and rapid scavenging of Tyr' and Trp' in proteins and free amino acids. Collectively, these results suggest that the activity of enzymes possessing a Tyr' or Trp' necessary for catalytic function may be significantly altered by 'NO, independent of its binding to protein-bound metal ions.

This is the first report documenting the rate constants for the direct reactions of 'NO with Tyr' and Trp' in amino acids, peptides and proteins. The very rapid rates of these reactions [$k = (1–2) \times 10^9$ $M^{-1} \cdot s^{-1}$], coupled with the increasingly apparent role of radical intermediates in biochemical processes, suggest that free amino acid and protein/enzyme radicals (Tyr' and Trp') are probable and important novel targets for 'NO *in vivo*. These findings may represent a novel paradigm with which to explain some of the biochemical functions associated with 'NO, including enzyme regulation and antioxidant activity.

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